Advances in Additive Manufacturing of Materials: Current status and emerging opportunities

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Lecture 38

3D (Bio)printing of GelMA hydrogels for neural tissue regeneration

welcome back to this NPTEL course on additive manufacturing. in today's lecture I will be covering 3D bioprinting of GelMa hydrogels for neural tissue regeneration. I have already introduced you the 3D printing and 3D bioprinting for your benefit I will be recapsulate 3D bioprinting essentially it is a layer by layer manufacturing of materials with biological system components. It can be cells, it can be biological macromolecules and so on which are contained with the synthetic materials during this layer by layer manufacturing. provided the biological macromolecules are not damaged and biological cells which are contained they remain their viability to the acceptable level or to the maximum extent after the layer by layer manufacturing then this entire process is called 3D bioprinting. I repeat it is a layer by layer which you have heard multiple times in this course then biological cells or macromolecules.

And GelMA is most widely investigated, widely studied hydrogel materials and this gelatin methacrylate, I think there is large number of research papers are published already and they are also under publications in different journals as I speak now. this particular slide was covered as part of the lecture on process science for 3D extrusion printing or 3D extrusion bioprinting. However, I think it is important to recapitulate here before I essentially describe this scientific case study. this is in the 3D extrusion printer, you have this nozzle, this nozzle is filled up with this hydrogel based bio ink.

This hydrogel based bio ink contains cells. And these hydrogel based bio ink also have these different hydrogel chains and then you have the cells. these cells are being colored in differently just to distinguish between cells which are mitochondrially viable which are stressed because it will experience this pressure like extrusion pressure. under this extrusion pressure whether the cells are viable or otherwise if the cells will experience significant stresses then cells become apoptotic and you know that cells either undergo necrosis or apoptosis and in this particular case it is more likely that cells will undergo necrosis because it is due to their viability is compromised because of the extrusion pressure induced stresses. Then when they will actually undergo and they will be extruded and this extrudability will be largely dependent on the rheological properties of this hydrogel based piving.

And this rheological properties depend on, rheological properties encompass viscosity, gel strength, yield properties, shear thinning at post printing recovery. Now shear thinning properties if you remember eta is equal to k gamma dot to the power n-1. gamma dot is the shear rate, eta is the viscosity, n is that exponent and this particularly if you plot eta versus gamma dot shear thinning behavior essentially shows that this kind of behavior. shear thinning and that is what is desired for successful 3D bioprinting. most often the research approach that most of the scientists they take is that first they optimize the 3D extrusion parameters for the biomaterial inks without biological cells.

then they define the printability window for that particular biomaterial ink and they try to use that similar window for 3D extrusion printing of cell laden biomaterial ink or cell containing biomaterial ink which scientifically is termed as bio ink this is the approach that people take and in the process they optimize the viscosity, gel strength, yield properties by essentially developing newer hydrogel formulations or by adding some of the carbonaceous materials to make it electro-active or some of the nano fillers to make it elastically stronger or stiffer if that is required for the particular clinical applications or targeted clinical applications. Then extrudability that is also important because extrudability depends on the extrusion pressure, their flow rate, nozzle type, nozzle size, printing temperature, nozzle height, printing time and filament stability. Other things that I have mentioned also in earlier lectures apart from shear thinning behavior, thixotropic properties. thixotropic properties is that if you plot the viscosity as a function of time, then also viscosity reduced over time. thixotropic behavior that is also important.

once the extrudability is established, then the next thing that is important for this particular 3D printing case is that what is the maximum height of the scaffold that you can print. and what is the different shapes like whether it is a kind of rectangular shape or cylindrical freestanding conduits those kind of structures can be built and whether the shape fidelity compliance is maintained. shafe fidelity compliance is maintained, so those things are to be addressed, once those things are addressed then you go for the 3D bioprinting and there you are essentially more concerned whether cells remain viable and then cell viability is not compromised to a large extent. while doing the 3D extrusion bioprinting and here viability, functionality and tissue maturation can be assessed at this stage extrudability and buildability, buildability what is the infill pattern, what is the layer height, line width, line spacing, cross linking mechanism and platform temperature those are important. And in this particularly key attributes whether during this pre-printing case while researchers they design new biomaterial inks they also look at what is the cellular functionality, maturation, biocompatibility of the ink.

even in the conventional cast conditions like 2D film. Their biophysical properties like mechanical properties and then scaffold degradation and so on and scaffold printability, viscoelasticity, shear thinning, gelation, cross-linking and buildability. GelMa the first thing that we did what is that while developing this kind of scaffolds, concentration dependent biophysical properties like when GelMa is being 3D printed with different concentrations like 3% GelMa to up to 20% GelMa how their properties they will vary. Now if you look at very carefully if I make this dotted line 3, 5, 10, 15, 20 the strength actually increases. with more concentration of GelMa and that must be due to the molar crosslinking density.

more the concentration of GelMa more is the crosslinking density and if you look at some of the modulus values 3% is very low, 5% is little bit increase and 5% to 10% to 15% to 20% and you can go the modulus values up to around 500 or 600 kilo Pascal . this particular so strength values is it kilo Pascal, modulus values is kilo Pascal and essentially strain values are percentage. as you can see that you know stress value so when you include when you go from low concentration GelMa to high concentration GelMa the essentially shifted to more towards the left. And molar cross-linking density as you see 3%, 5%, 10%, 15%, 20% molar cross-linking density increases not in a linear fashion but suddenly it increases more I would say more like a parabolic manner. if you fit this data you will get a second order polynomial kind of a curve.

Now, gelma concentration is important but most often since the higher concentration gelma provides the better properties in terms of strength and modulus, many researchers they have investigated that higher concentration gelma and the number of research papers are relatively less in the lower concentration gelma. when we started our research more than 6-7 years ago on this 3D extrusion printing or for soft tissue regeneration, we largely

concentrated in our group or more on the low concentration gelma like 5% gelma or 7.5% gelma and so on. many of the scientific case studies in this particular case particularly for 3D extrusion printing will largely focus on the low concentration gelma. in this low concentration gelma what you see here also the swelling properties as you know this hydrogels by definition it contains hydrophilic polymeric macromolecular chains with the ability for significant water molecule retention that is the definition of hydrogel you have learned in some of the earlier lectures.

in this particular case, if you go from 3% to 20% Gelma, you will certainly see that swelling curve essentially goes down. that means maximum swelling is reduced but qualitatively it shows identical trend. volume change that essentially reduced. Now, mass change, mass remaining like enzymatic degradation case, these also shifted towards more in this way with larger concentration of gelma. what you see here that after 5 days still if you formulate a new hydrogel composition like 5% GelMa with 1 MC and 1 H like hydrogel.

hydroxyapatite and modified carboxymethyl cellulose, you can have almost like 30% of the scaffold still remain after 5 days of enzymatic degradation. Whereas 3% or 5% gelma everything gets degraded even within 1 day of the enzymatic degradation studies like where they use this collagenous solution. this is like more kind of correlation between biophysical properties and crosslinking density because I must mention that in gelatin methacrylate when you do this 3D printing after that 3D printing you do physical crosslinking. to allow physical crosslinking to happen under the UV exposure you essentially mix irga cure with the hydrogels. that this photo crosslinker will facilitate the physical crosslinking process.

And if you plot this elastic modulus as a function of molar crosslinking density, it shows a perfectly linear plot, y is equal to mx plus c type of plot. If you look at the compressive strength with the molar crosslinking density, It shows kind of not like a linear but also kind of exponential type because there is a E function exponential function. Swelling ratio it shows asymptotic behavior like it goes down and the same is the degradation rate asymptotic ratio. Now all these 4 properties are dependent clearly dependent on the molar crosslinking density and that is the way one has to analyze this gelma properties. often you will notice in the research papers or publications people always put the gelma concentration at the x-axis that is not correct because the physical properties which will influence this strength and elastic modulus and so on those must be some physical properties and there as it has been identified here it is the molar cross-linking density that is the parameter which influences all these properties.

The microstructure, now when you crosslink this microstructure that you will notice that how the pore 3 dimensional pore architecture that develops and these 3 dimensional pore architecture if you see if you go from 3% to 5 to 20% the pore cross sectional area subsequently reduces. and volume percent porosity also is reduces to around 30%. 3% Gelma to 20% Gelma. Rheological properties as I mentioned to you earlier in some of the earlier lectures that parallel plate rheometer. Parallel plate rheometer is most extensively used to characterize the rheological properties of this kind of soft hydrogel.

And as part of the scientific case study, we have used the same parallel petriometer. We have taken different hydrogel compositions and then measured the viscosity, measured G prime and G double prime. G prime is your storage modulus. G double prime is your loss modulus and tan delta is essentially G double prime by G prime. that is the tan delta loss factor.

these properties how we measured and then what is the relevance that? were covered when I gave this lectures

on the rheological properties and fundamentals of rheological properties or I have introduced to the different rheological properties. these kind of plots are to be analyzed to define shear thinning properties for example. this is eta and this is gamma dot. As you see, shear thinning properties has to be analyzed by eta is equal to k gamma to the power n-1 and in the log scale, this is in the log scale, this is also in the log scale. if you take log scale so it becomes more like a linear log eta is equal to log k plus n-1 into log gamma dot then if you plot log eta versus log gamma dot you will get essentially the slope is nothing but n-1.

you must obtain a linear curve that we got experimentally and why 21 degree Celsius because that is the 21 degree Celsius we find that is the optimum printability window and that we have got from the measurement of the rheological properties in the temperature sweep or as a function of temperature. these are the different gelma concentration like 3% gelma to 20% gelma and you see that as you increase or as you formulate the newer composition like 5% gelma, 1% modified carboxymethyl cellulose, 1% hydroxyapatite. their shear thinning properties, their viscosity is lowered down quite a bit from 10 to the power 8 for the 3% gelma or 5% gelma or 20% gelma but compared to that it is lowered quite a bit. Whereas if you go to higher and higher gelma concentrations, your viscosity actually increases, It makes the hydrogel more viscous. Now on the left hand side G prime and G double prime as a function of temperature and you can see why 21 degree Celsius in the temperature sweep you can see that you know how this G prime and G double prime goes through a transition and on the left hand side this is a temperature sweep and in the bottom G prime and G double prime as a function of shear stress and shear stress if you see at 21 degree Celsius up to large shear stress in the 20% gelma and this is the 20% gelma, this is the G double prime and these are 20% gelma G prime and there are also this is the 3% gelma, so this is like G prime G double prime, so as you increase the gelma concentration you see there is a upward increase in the G prime and G double prime at any given shear stress value.

And the other thing that you can see that if you go from 3% to gelma to higher percent of gelma essentially your gel exhibits stable viscoelastic properties even up to a large shear stress which is not happening or which is not possible for the low concentration of gelma. now you see that why low concentration, why high concentration or why relatively medium concentration of gelma which were used in the 3D extrusion printing. This was more in the science aspect. Now, when you see that 3% GelMa it goes to 5% and then if you get also 1% carboxymethylcellulose and 1% carboxymethylcellulose hydroxyapatite, 3% GelMa it is no good right because you know there is no safe fidelity.

5% GelMa also no good. But when you add carboxymethyl cellulose and further you add hydroxyapatite you can see that color also appearance also changes and you can go up to very large structures and you can go up to 45 layers of the structures the printing speed is 10 millimeter per second each layer height is 0.3 millimeter and printing temperature is 21 degree Celsius. 3% GelMa is no good, 20% GelMa is good but I do not think printing resolution is that good. as I said that our motivation was to work on the intermediate or low concentration of GelMa and we demonstrated that in the low concentration of GelMa also you can essentially build self-fidelity compliance structures that is important. Now in terms of the filament collapse test.

or filament fusion test when you go from 3 to 20% GELMA suddenly your filament is more and more stable. But there is also sagging in the 10% GelMa and 15% GelMa and you can see this 5% GelMait is complete sagging but when you make this hydroxyapatite laden and carboxymethyl cellulose which has been added to 5% GelMa it shows very stable filament stable behavior during the filament collapse test study. Now, when we develop this or formulated this kind of scaffolds, the first thing we have used, we have used the cell culture study using bone marrow derived mesenchymal stem cells, what is stem cells that were already introduced to

you before in the lecture on introduction to biological system. differentiation what is differentiation that is the differential gene expression. essentially stem cells which is unspecialized cells.

transforms or it undergoes differentiation to more mature functional cell type and here we are wondering whether the stem cells can undergo to more bone cells, And here we have used the different gelma concentration 5% gelma and to 5% gelma 1% carboxymethyl cellulose to 1% hydroxyapatite. And human osteoblasts also that is the bone forming cells in the differentiation media and if you see this alkaline phosphatase activity these are like activities which are essentially measured to see whether their differentiation process is progressing and more the ALP activity means that these cells differentiated cells will have attributes more bone like cells. And these HMSCs were cultured in non-differentiating media as well as the differentiating media and this is the fluorescence microscopy images. The fluorescence microscopy images anytime you will find the blue circles, these are like nucleus, green ones are essentially cytoskeleton or actin filaments, these are strained before the fluorescence microscopy observations. one can clearly see that whether HMSCs they express more ALP after growing them on this different kind of low to medium or low concentration of gelma or modified gelma hydrogels with carboxymethyl cellulose and hydroxyapatite.

Next few slides what I am going to do, I am going to start showing you the case study for the peripheral nerve regeneration. what you see is that you have a human brain. total nervous system is composed of the central nervous system CNS and peripheral nervous system PNS. Central nervous system essentially means it contains brain and spinal cord that is the two important part of the central nervous system. brain and spinal cord as you know they are essentially determine most of the human activities, whether perception or processing of sensory stimuli, control awareness, movement, thinking, speech, programming of spinal cord reflexes, control of internal environment to maintain homeostasis.

Peripheral nervous system that send out information from different areas. of your body back to your brain or carrying out commands from your brain to various parts of your body. In many injuries or during some of the surgeries, if your peripheral nerves are cut or if a patient's peripheral nerves are injured or they need to be locally cut or it undergoes some kind of a neurectomy process then what you do that you can use these nerve grafts and then question is whether this kind of cylindrical conduit structures which will have neural tissue like properties can be 3D printed. and can be implanted in patients to restore the neurological functionality. if you look at this particular peripheral nervous system or peripheral nerves, if you look at that how what are the things you have, you have a this is the cylindrical conduit structures.

And then you have adipose tissue, you have epineurium, perineurium, endoneurium and also you have schwan cells and axons. these are like different kind of neural cells are there and you must have vasculature like artery and vein. without vasculature you know the tissue will not receive the oxygen supply and other nutrients supply and so on. now what you see here, these particular things if you blow up, you can see that in this particular neuron structures.

you have dendrites. You have a nucleus, small nucleus and then in this extended tail and this extended tail, you have myelin sheath, you have schwan cells, you have axon terminals. these are like classical and this is the large cell body. this is the axons and this is the dendrites with nucleus and so on. This is the cell body.

you have a cell body, then you have axons. And often people measure axonal growth of the neural cells or if the stem cells undergo neurogenesis then they are interested to see that whether axonal growth has taken place.

And in a matured neural cells this axonal growth would be very significant and this has large aspect ratio because this length of the axons is much much larger compared to the cell body. more into this clinical aspects this neurons can be bipolar, pseudo unipolar, multipolar and pyramidal cells. these are like different type of peripheral neurons that in human body can have. let me spend little bit time here to give you more clinical relevance before we move on to the 3D extrusion bioprinting.

as I said during the surgery or during some injury if there is peripheral nerve injury. in this peripheral nerve injury what happens that this locally there is a gap between the two ends of the nerves and this gap needs to be filled up. And these nerve gaps you have this nerve injury and this nerve injury it can be done either by nerve suturing if the gap is very small or it can be nerve grafting, autograft you are talking about we are talking about that means some part of the nerves cut from another location of the same patients and grafted back to the peripheral nerve injured locations. And you can give external stimuli like laser radiation, electric charge or ultrasound and so on for this treatment of the nerve injury conventional treatment. More advanced treatment we are talking about is a nerve guidance conduit.

These are proximal nerve stump, these are distal stump, this particular between this particular region you need to have this nerve repair conduit. this nerve repair conduits if it is 3D extrusion printed, if it is pores and if it facilitates this axonal regeneration to grow through this entire scaffolds to bridge this gap and therefore neurological functionality in this particular region like which I am tracing here with my pen if that can be restored And if that is restored means this one cells must be migrated and action can be regenerated and then that gives rise to this entire functionality of the 3D bioprinted scaffolds. now you can see that what is the concept of critical size defects. Now this length can be somewhere around 20 millimeter length. And this diameter can be outer inner diameter can be some of these like 5 into 5, 4 to 6 millimeter in diameter.

while making this 3D extrusion printing or extrusion bioprinting, we have to make sure that we are able to fabricate this kind of nerve guidance conduits and that can be clinically tested further. I will come back to you on that more 3D extrusion bioprinting to show you how we have formulated different hydrogel compositions to fabricate these nerve guidance conduits in the 3D extrusion bioprinting route.